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Pilot study: potential transcription markers for adult attention-deficit hyperactivity disorder in whole blood

Grünblatt, Edna ; Geissler, Julia ; Jacob, Christian P ; Renner, Tobias ; Müller, Maja ; Bartl, Jasmin ;
Gross-Lesch, Silke ; Riederer, Peter ; Lesch, Klaus-Peter ; Walitza, Susanne ; Gerlach, Manfred ;
Schmitt, Angelika

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Pilot study: potential transcription markers for adult attention-deficit hyperactivity disorder in whole blood

Edna Grünblatt · Julia Geißler · Christian P. Jacob · Tobias Renner ·
Maja Müller · Jasmin Bartl · Silke Gross-Lesch · Peter Riederer ·
Klaus-Peter Lesch · Susanne Walitza · Manfred Gerlach · Angelika Schmitt

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Abstract Attention-deficit hyperactivity disorder (ADHD) is a common behavioural disorder that affects not only children and adolescents but also adults; however, diagnosis of adult ADHD is difficult because patients seem to have reduced externalized behaviour. ADHD is a multifactorial disorder in which many genes, all with small effects, are thought to cause the disorder in the presence of unfavourable environmental conditions. Therefore, in this pilot study, we explored the expression profile of a list of previously established candidate genes in peripheral blood samples from adult ADHD subjects ($n = 108$) and

compared these results with those of healthy controls ($n = 35$). We demonstrate that combining the gene expression levels of dopamine transporter (*SLC6A3*), dopamine D5 receptor, tryptophan hydroxylase-1, and *SNAP25* as predictors in a regression model resulted in sensitivity and specificity of over 80 % (ROC: max $R^2 = 0.587$, AUC = 0.917, $P < 0.001$, 95 % CI: 0.900–0.985). In conclusion, the combination of these four genes could represent a potential method for estimating risk and could be of diagnostic value for ADHD. Nevertheless, further investigation in a larger independent population including different subtypes of ADHD (inattentive, hyperactive, or combined type) patients is required to obtain more specific sets of biomarkers for each subtype as well as to differentiate between child, adolescent, and adulthood forms.

Edna Grünblatt and Julia Geißler have contributed equally.

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E. Grünblatt (✉) · S. Walitza
Hospital of Child and Adolescent Psychiatry, University
of Zurich, Neumünsterallee 9, 8032 Zurich, Switzerland
e-mail: edna.gruenblatt@kjp.dzh.ch

S. Walitza
e-mail: susanne.walitza@kjp.dzh.ch

E. Grünblatt · J. Geißler · C. P. Jacob · M. Müller · J. Bartl ·
S. Gross-Lesch · P. Riederer · K.-P. Lesch · A. Schmitt
Institute of Molecular Psychiatry, Clinic and Polyclinic for
Psychiatry, Psychosomatics and Psychotherapy, University
Hospital of Würzburg, 97080 Würzburg, Germany
e-mail: E_Geissler_J@klinik.uni-wuerzburg.de

C. P. Jacob
e-mail: Jacob_C@klinik.uni-wuerzburg.de

M. Müller
e-mail: majumueller@yahoo.de

J. Bartl
e-mail: E_Bartl_J@klinik.uni-wuerzburg.de

S. Gross-Lesch
e-mail: Gross_S1@klinik.uni-wuerzburg.de

P. Riederer
e-mail: Peter.Riederer@mail.uni-wuerzburg.de

K.-P. Lesch
e-mail: kplesch@mail.uni-wuerzburg.de

A. Schmitt
e-mail: schmitt_A3@klinik.uni-wuerzburg.de

T. Renner · M. Gerlach
Institute of Molecular Psychiatry, Clinic and Polyclinic for
Psychiatry, Psychosomatics and Psychotherapy,
University Hospital of Würzburg, 97080 Würzburg,
Germany
e-mail: Renner@kjp.uni-wuerzburg.de

M. Gerlach
e-mail: manfred.gerlach@uni-wuerzburg.de

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Introduction

Attention-deficit hyperactivity disorder (ADHD) is one of the most common behavioural disorders in child and adolescent psychiatry (prevalence variance of 5–12 %) (Faraone et al. 2003; Polanczyk et al. 2007), while recently it was demonstrated in adults with a prevalence variance of 3–6 % (Klassen et al. 2010; Simon et al. 2009; Lara et al. 2009). The disorder is characterized by impaired attention, motor hyperactivity, and increased impulsivity (Haavik et al. 2010; American Psychiatric Association A 2000). Adult ADHD is a well-characterized condition, although it is generally accepted that the onset of ADHD usually occurs in childhood (Biederman 2005; American Psychiatric Association A 2000). The diagnosis of and treatment for adult ADHD can be challenging since hyperactive symptoms and externalized behaviours tend to wane with age, making diagnosis difficult (Klassen et al. 2010).

Despite evidence pointing towards a primarily genetic basis for ADHD, no specific genetic variants are yet known (Wood and Neale 2010; Banaschewski et al. 2010; Franke et al. 2009). The candidate genes explored so far confirm only small-to-moderate effects, with odds ratios (OR) ranging from 1.03 to 1.83 (Faraone and Mick 2010). The most frequently studied genes affecting the susceptibility to ADHD are the dopamine D4 receptor (*DRD4*), *DRD5*, dopamine transporter (*SLC6A3*, *DAT1*), 25-kDa synaptosomal-associated protein (*SNAP25*), serotonin transporter (*SLC6A4*, *SERT*), and tryptophan hydroxylase 2 (*TPH2*) (Faraone and Mick 2010; Gizer et al. 2009), but all seem to have mild effects. Additionally, environmental factors such as stress and prenatal alcohol or nicotine consumption could also influence the ADHD phenotype and prevalence, which are part of many components regarded as gene \times environmental interaction (Nigg et al. 2010). Therefore, there is probably no single gene indicating the presence of ADHD, but rather polygenetic markers that in combination increase the risk of the disorder. Such an approach, using a wide array of parameters, is referred to as biomarkers.

Biomarkers can be used as diagnostic tools to identify or stage disease, as indicators of disease prognosis or predictors, or to monitor clinical response to therapy. As defined by the Biomarkers Definitions Working Group, a biomarker is ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working Group 2001). Consequently, in this pilot study, we explore a group of candidate genes comprising the

described risk genes known in the literature (Faraone and Mick 2010; Gizer et al. 2009), and corticotropin-releasing factor binding protein (*CRHBP*) was selected as a response to stress, which is also thought to be a risk factor for ADHD, to assess their expression profile in peripheral blood samples taken from adult ADHD subjects compared with samples from healthy controls. Such concept was previously reported in which transcriptional profiling resulted in identification of several candidate genes in blood and brain, especially for schizophrenic patients (Glatt et al. 2005; Tsuang et al. 2005; Mehler-Wex et al. 2006; Noori-Daloui et al. 2010; Taurines et al. 2010) and Alzheimer’s and Parkinson’s disease patients (Grünblatt et al. 2009, 2010; Scherzer et al. 2007).

The concept of peripheral blood cells as a ‘window’ into the central nervous system (CNS) was proposed nearly a decade ago (Gladkevich et al. 2004; Burczynski and Dörner 2006), whereas CNS versus peripheral expression has proven to moderately correlate (Sullivan et al. 2006; Cai et al. 2010). We hypothesized that none of the biomarkers would individually provide high sensitivity and specificity; therefore, we used the best known candidates in a combined gene expression analysis and a comprehensive regression model.

Methods

Subject recruitment

Patients and controls were recruited and phenotypically characterized by a team of experienced psychiatrists at the outpatient units of the Department of Psychiatry, Psychosomatics, and Psychotherapy, University of Würzburg, Germany, according to the DSM-IV criteria (American Psychiatric Association A 2000; Jacob et al. 2007). The diagnosis of childhood and youth manifestation of ADHD was retrospectively assessed with the DSM-IV symptom list for ADHD, which was used as a structured clinical interview. Additional information from parents and school report cards was included if available. Adult symptomatology was assessed with the DSM-IV symptom list for ADHD. To ensure diagnostic validity, informative input from partners, relatives, and friends was also collected. Co-morbidity was assessed with the structured clinical interviews of axis I (SCID I) disorders (First et al. 1997). Exclusion criteria were autistic disorder, psychotic disorder, bipolar affective disorder, history of epilepsy, other neurologic disorder, severe chronic medical condition, IQ < 80 (Jacob et al. 2007). Additionally, detailed information on medication, alcohol abuse, and smoking habits was collected. Control subjects underwent diagnostic procedures similar to those of the patients to exclude any other

psychiatric disorders. Controls were recruited through local newspaper ads. The subjects provided informed consent according to the Helsinki Declaration. The study protocol was approved by the local ethics committee of the Universities of Würzburg, Germany. Written informed consent was obtained from all subjects or their primary caregivers.

One hundred and eight patients diagnosed with ADHD (46 men and 62 women) and 35 healthy controls (19 men and 16 women) were included in this study (Table S1, supplemental data). All subjects were with German ancestry that ensured a homogenous sample analysis. The gender distribution was similar in both diagnostic groups ($P = 0.247$, Table S1), whereas the mean age was significantly older in the control group (controls = 39.6 ± 9.49 years, ADHD = 34.7 ± 10.52 years; $P = 0.009$). The most frequent lifetime co-morbidities were substance-related disorders (alcohol, 11.1 %; cannabis, 22.2 %), and other co-morbidities were present in 11.1 % of patients. In ADHD patients, the combined subtype was diagnosed in 70.4 % ($n = 76$), whereas 21.3 % ($n = 23$) were primarily inattentive, and 5.5 % ($n = 6$) were primarily hyperactive. Whole blood was collected with the *PAXgene*TM Blood RNA system (Becton–Dickinson GmbH, Heidelberg, Germany) and EDTA-blood tubes. Blood samples were frozen at -20°C until further processing for total RNA extraction, whereas fresh blood was processed immediately for DNA isolation.

Genomic DNA extraction

DNA was prepared from 2 ml EDTA-blood using proteinase K as described previously (Grünblatt et al. 2005). The DNA was then aliquoted into cryo-Vials (Nunk GmbH, Wiesbaden, Germany) and frozen at -70°C until processing.

Genotyping

Genotyping was conducted as previously described for *SNAP25* +2015 (rs6077690), *SNAP25* +80609 (rs363006), *SNAP25* -523 (rs6039769) (Renner et al. 2008), serotonin transporter linked polymorphic region (*HTTLPR*; *SLC6A4* tri allelic) (Lesch et al. 1996; Hu et al. 2005), *TPH2* SNP25 (rs4570625) (Walitza et al. 2005), *SLC6A3* 3'UTR VNTR, and *DRD4* exon 3 VNTR (Walitza et al. 2008).

Total RNA extraction

Total RNA was prepared with the *PAXgene*TM Blood RNA Kit 50 (PreAnalytiX, Qiagen and BD, Germany). RNA isolation reagents were prepared from 0.2 μM filtered,

diethyl pyrocarbonate (DEPC)-treated water (Fermentas Inc., Hanover, MD, USA) throughout the isolation procedure. Total RNA samples were spectrophotometrically scanned (Experion, BioRad Co., Hercules, CA, USA) from 220 to 320 nm; the A260/A280 of total RNA was typically >1.9 .

Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR was used for the following 7 genes (Table S2, supplemental data): *SLC6A3*, *DRD4*, *DRD5*, *TPH1* (variant 1 was tested in the peripheral samples since variant 2 [*TPH2*] is not expressed in the peripheral blood but only in the CNS), *SLC6A4*, *SNAP25*, *CRHBP*; additionally, 6 reference genes were used as internal controls. Total RNA (500 ng) from each sample was reverse transcribed with the random hexamer and oligo-dT primer mix using iScript (BioRad Co., Hercules, CA, USA). Quantitative real-time PCR was performed in the iCycler iQ system (BioRad Co., Hercules, CA, USA) as described previously (Grünblatt et al. 2009). Real-time PCR was subjected to thermocycler amplification as described previously (Grünblatt et al. 2009). All PCR reactions were run in duplicate. The amplified transcripts were quantified using the comparative C_T method analysed with the BioRad iCycler iQ system program. Gene expression levels were normalized to the 6 reference genes according to GeNorm (Vandesompele et al. 2002). Data were analysed with Microsoft Excel 2000 to generate raw expression values.

Statistical analysis

Because of non-normal data distribution, parametric procedures including all potential moderating variables could not be employed; therefore, we tried to control for the influence of age on individual mRNA levels by looking at the correlation between age and gene expression. The relationship between genotype, diagnosis, and gender was explored with a chi-squared test on 2 (diagnosis) \times 3 (genotype) cross-tabs both with gender as a layer variable for each gene and for both genders combined. For analysis of our main variable of interest—gene expression differences between diagnostic groups—non-parametric Mann–Whitney *U* tests for differences in mean rank between ADHD subjects and controls were performed. For those genes returning significant main effects of diagnosis, we also checked for the influence of genotype on gene expression within each diagnostic group.

Assuming a combination of markers would yield the best prediction, we employed binary logistic regression to first assess the diagnostic utility of the expression of our

candidate genes for the ability to differentiate between ADHD patients and healthy controls. To that end, we entered those genes showing differential expression between ADHD and controls as well as the potentially confounding variables age and gender into the model. We applied a filter, excluding cases rated as outliers on any one of the respective RNA levels. Outliers were identified using box plots, with values more than 1.5 and 2 box-lengths away from the box ends are characterized as such. In total, 19 samples had to be excluded, because they were classified as outliers on the expression level of at least one gene. The predicted probabilities from significant predictors from this whole model were then ultimately used for building a receiver operating characteristic (ROC) curve and assessing sensitivity and specificity. Since odds ratios (OR) were extremely large due to the small unit of the detected gene expression levels (e.g. 6666.13 for TPH1), the original mRNA values were multiplied by a factor of 100. Significances remained unchanged by this procedure. The significance level was set as 0.05 (nominal P values). All computations were completed using the statistical computing environment, PASW Statistics 18.

Results

All genotypes were in Hardy–Weinberg equilibrium. For exact genotype distributions, see supplementary Table S3. From the results of the different genotypes, we detected no association between ADHD and any of the polymorphisms.

Except for lower *TPH1* levels ($r = -0.174$, $P = 0.039$) being weakly associated with older age, no significant correlations between age and any of the genes were observed (all $P > 0.105$; for details, see supplementary data, Tables S4 and S5).

The dopaminergic system showed the most prominent differences between ADHD patients and controls, with patients having elevated *SLC6A3* expression ($P = 0.001$), and both *DRD4* and *DRD5* showed higher expression levels in ADHD patients compared with controls ($P = 0.005$ and $P < 0.001$, respectively; Table 1; supplementary Figure S1). Furthermore, there was a notable increase in *SNAP25* in samples from ADHD subjects compared with controls ($P = 0.01$). With regard to the serotonergic genes, no difference was found for *SLC6A4* ($P = 0.696$); however, ADHD subjects had higher expression of the rate-limiting enzyme *TPH1* mRNA ($P < 0.001$). *CRHBP* mRNA was the only gene expressed at lower levels in ADHD cases ($P = 0.037$). Within the diagnostic groups, no influence of genotype on gene expression was observed (Table 1; Supplementary Figure S2).

For our first binary logistic regression model, we used a forward stepwise approach, in which all variables were successively entered. In this case, we used the expression levels of all candidate genes (*SLC6A3*, *DRD4*, *DRD5*, *TPH1*, *SNAP25*, and *CRHBP*) along with age and gender as potential predictors. Concerning multicollinearity, all correlations of variables within this model were below 0.510. The whole model returned the following significant regressors: *DRD5* ($P = 0.011$, OR = 1.40, 95 % CI: 1.08–1.81), *TPH1* ($P < 0.009$, OR = 1.10, 95 % CI: 1.02–1.17), and *SNAP25* ($P = 0.021$, OR = 1.07, 95 % CI: 1.01–1.13) and a trend-level contribution for *SLC6A3* ($P = 0.069$, OR = 1.06, 95 % CI: 1.00–1.13) (Supplementary Table S6). The receiver operating characteristic (ROC) curve was built using these four genes (Fig. 1: max $R^2 = 0.587$, AUC = 0.917, $P < 0.001$, 95 % CI: 0.900–0.985). Combining gene expression levels of *SLC6A3*, *DRD5*, *TPH1*, and *SNAP25* as predictors in a regression

Table 1 Gene expression patterns in attention-deficit hyperactivity disorder (ADHD) patients versus control subjects and genotype effects

	ADHD subjects (mean \pm SD)		Controls subjects (mean \pm SD)	P	Genotype effects (P values)	
					ADHD	Controls
<i>SLC6A3</i>	0.37 \pm 0.19	>	0.25 \pm 0.09	0.001	0.537	0.783
<i>DRD4</i>	0.36 \pm 0.27	>	0.24 \pm 0.14	0.005	0.684	0.206
<i>DRD5</i>	0.13 \pm 0.09	>	0.07 \pm 0.04	<0.001	N/A	N/A
<i>CRHBP</i>	0.17 \pm 0.11	<	0.21 \pm 0.12	0.037		
<i>SNAP25</i>	0.41 \pm 0.29	>	0.24 \pm 0.08	0.001	0.311	0.126
<i>TPH1</i>	0.44 \pm 0.30	>	0.19 \pm 0.06	<0.001	0.800	0.598
<i>SLC6A4</i>	0.16 \pm 0.09		0.17 \pm 0.09	N/S	N/D	N/D

N/A data not available since no genotyping was conducted, N/S not significant, N/D not done (since the main result was not significant), *SLC6A3* dopamine transporter (DAT), *DRD4* dopamine D4 receptor, *DRD5* dopamine D6 receptor, *CRHBP* corticotropin-releasing factor binding protein, *SNAP25* synaptosomal-associated protein of 25 kDa, *TPH1* tryptophan hydroxylase, *SLC6A4* serotonin transporter

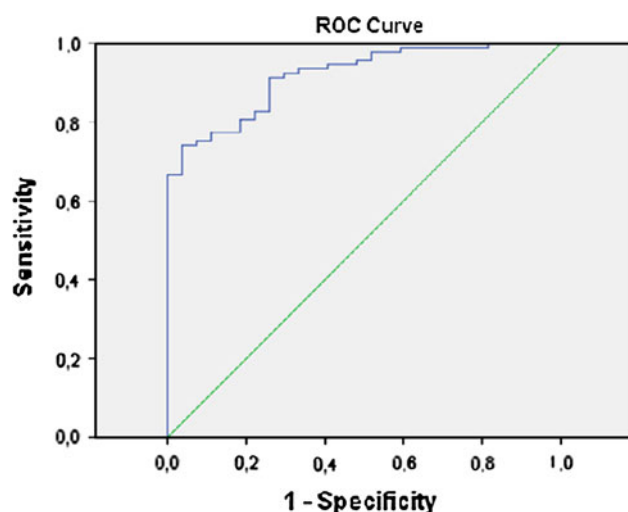


Fig. 1 Receiver operating characteristic (ROC) curve for the candidate markers, dopamine transporter (*SLC6A3*), dopamine D5 receptor (*DRD5*), tryptophan hydroxylase 1 (*TPH1*), and synaptosomal-associated protein of 25 kDa (*SNAP25*) mRNA

model, 80 % of cases could be correctly classified as ADHD subjects or controls. Any other combination with other genes or with three or two of the above four resulted in a worse prediction of ADHD compared with this four-gene combination. We achieved sensitivity and specificity of over 80 % (e.g. sensitivity of 81 % and specificity of 82 % at a cut-off of 0.69) according to the whole-model ROC analysis. The combined approach was superior to any individual variables predictive ability, where separate logistic models returned the following results: *SLC6A3* (AUC = 0.694, sensitivity = 0.696, specificity = 0.645), *DRD5* (AUC = 0.749, sensitivity = 0.752, specificity = 0.625), *SNAP25*

(AUC = 0.689, sensitivity = 0.637, specificity = 0.618), and *TPH1* (AUC = 0.812, sensitivity = 0.776, specificity = 0.706) (Table 2).

Discussion

We showed that a combination of expression patterns for four ADHD-associated genes in the peripheral blood of adult ADHD patients could be a potential method for estimating risk and have possible diagnostic value for ADHD. Since ADHD is a heterogeneous disorder with a high comorbidity rate, a complex diagnostic tool with more than one marker was expected (Wallis 2010). We show that a combination of expression levels of four genes, *SLC6A3*, *DRD5*, *TPH1*, and *SNAP25*, provide relatively high sensitivity and specificity, indicating the potential use of this combination of biomarkers to identify the risk of ADHD in adults. Three of the genes studied were unaffected by age, whereas *TPH1* showed a very mild decrease in expression with age. Recently, in a study for mRNA alterations in whole blood from child and adolescent ADHD compared with autism spectrum disorder (ASD) and with controls, no group differences were found for *TPH1* mRNA expression (Taurines et al. 2011). Still, *TPH1* expression of the ADHD group ($n = 51$) was slightly higher than that of the ASD ($n = 26$) or the control ($n = 39$) group (Taurines et al. 2011), which might indicate the beginning of alterations with age for ADHD group. As an additional parameter, we analysed the association of the genotypes for the same genes studied with ADHD. We observed no overall association between genotype and ADHD for any of the genes tested; moreover, we checked for an additional influence of genotype on the mRNA levels of those genes found to be differently expressed in patients and controls; however, this also showed no difference. Since transcript expression seems to be influenced rather by multiple factors such as multiple genetic polymorphism as well as environmental factors, the notion that multiple transcript analysis would be necessary to identify ADHD cases is pointed out.

The four candidate genes identified as the best biomarkers in this study were previously described in other ADHD association studies (Stergiakouli and Thapar 2010; Gizer et al. 2009). The most consistent evidence for an association with ADHD exists for the *DRD5* gene microsatellite (Squassina et al. 2008) and the *SLC6A3* gene variable tandem repeat (VNTR) at the 3' UTR (Stergiakouli and Thapar 2010; Gizer et al. 2009). In contrast, very weak individual associations were reported for *TPH1* (Gizer et al. 2009; Johansson et al. 2010) and *SNAP25* (Gizer et al. 2009; Forero et al. 2009; Kim et al. 2007), but the 3' UTR of the latter was significant in a recent meta-analysis (Gizer et al. 2009). Each of these genes often showed a

Table 2 Individual logistic regression models for each biomarker

	<i>P</i> value	OR (95 % CI)	AUC
Age	0.018	0.95 (0.92–0.99)	0.646
Gender	0.229	0.63 (0.29–1.35)	0.558
<i>SLC6A3</i>	0.003	1.06 (1.02–1.10)	0.694
<i>DRD4</i>	0.015	1.04 (1.01–1.06)	0.656
<i>DRD5</i>	0.001	1.21 (1.08–1.35)	0.749
<i>CRHBP</i>	0.074	0.97 (0.94–1.00)	0.381
<i>SNAP25</i>	0.002	1.05 (1.02–1.08)	0.689
<i>TPH1</i>	<0.001	1.09 (1.05–1.14)	0.812
<i>SLC6A4</i>	0.705	0.99 (0.95–1.04)	0.478

AUC area under the curve (for gender 1 = male, 2 = female), OR odds ratio, *SLC6A3* dopamine transporter (DAT), *DRD4* dopamine D4 receptor, *DRD5* dopamine D6 receptor, *CRHBP* corticotropin-releasing factor binding protein, *SNAP25* synaptosomal-associated protein of 25 kDa, *TPH1* tryptophan hydroxylase, *SLC6A4* serotonin transporter

OR higher value refers to attention-deficit hyperactivity disorder (ADHD) cases, $P < 0.05$ (uncorrected)

very modest genetic effect on the risk of ADHD (meta-analysis: SLC6A3 10 repeats OR = 1.1, 95 % CI: 1.03–1.17; DRD5 5' Flank OR = 1.22, 95 % CI: 1.10–1.36; TPH1 intron 6 OR = 0.99, 95 % CI: 0.89–1.10; SNAP25 3' UTR OR = 1.15, 95 % CI: 1.01–1.31) (Gizer et al. 2009). Interestingly, SLC6A3 seems to have an opposite effect in persistent adult ADHD compared with childhood ADHD (Franke et al. 2010). Therefore, the need for sensitive biomarkers that in combination achieve a much higher OR than the ones investigated to date is necessary. In our study, we combined multiple genetic markers with relatively good success, which confirms the potential of this strategy.

A limitation of this study is the small sample size; however, since this was planned as an exploratory study, the sample size still pointed to the possible candidate genes that should be than confirmed in the future. Additionally, the different co-morbidities existing in our population might have affected the results, which we could not control for properly because of the limited group size. Since adult ADHD is highly co-morbid with other ICD diagnoses (Wallis 2010), there might be common pathways for the array of these disorders that still provides a good diagnostic tool. It will be necessary to look at subgroups in future studies with a larger population and to analyse markers for a specific diagnosis. Furthermore, a larger population would enable us to incorporate the different pharmacological treatments to rule out any effects of these on gene expression. The biggest limitation might be the fact that the populations we examined and determined the test's sensitivity and specificity from were extensively evaluated by trained professionals, and the subjects included in the study had either a very clear-cut case of ADHD or were completely healthy. However, in real-life patients, presentations are often a lot more ambiguous, making the development of a highly sensitive and specific test a top priority. In that case, our markers might do less well, so results need to be validated with a broader spectrum of ADHD phenotypes and severities, and the predictive value needs to be determined.

In conclusion, we demonstrate the potential of peripherally expressed genes as biomarkers that could be used to diagnose ADHD. Further investigation of additional candidate transcripts in a larger independent population including different ADHD sub-groups (inattentive, hyperactive, and combined type) and comparing them not only with healthy controls but also with other psychiatric disorders is required to obtain more specific sets of biomarkers for each sub-disorder and to differentiate between childhood, adolescent, and adult forms of ADHD.

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Conflict of interest The authors have no conflicts of interest to declare.

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